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Receptor-Mediated Endocytosis of Decorin: Involvement of Leucine-Rich Repeat Structures

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Decorin, a small dermatan sulfate proteoglycan, is characterized by a core protein with central leucine-rich repeat structures and a single glycosaminoglycan chain. It is catabolized by receptor-mediated uptake and subsequent intralysosomal degradation. In the present study, the localization of the receptor binding site(s) along the core protein was investigated. Various recombinant decorin fragments were consistently able to inhibit the endocytosis of wild-type decorin. The most potent inhibitory peptides were those which encompassed the central Leu125–Val230 region, i.e., the fifth to eighth leucine-rich repeat, or at least a part of it. The peptide Leu125–Val230 bound directly to the 51-kDa endocytosis receptor, and Fab fragments of antibodies against this peptide inhibited the endocytosis of decorin in a dose-dependent manner. Decorin constructs expressed in human 293 cells and comprising the full-length coding region or lacking sequences N- and/or C-terminally of the Leu125–Val230 region were all endocytosed with similar clearance rates. These data suggest that the N- and C-terminal domains of the core protein are not required for endocytosis. The receptor binding site is rather represented by contiguous leucine-rich repeat structures of the central part of the core protein. This conclusion is supported by competition experiments with biglycan, a structurally related small proteoglycan. © 1998 Academic Press

Key Words: **biglycan; endocytosis receptor; core protein; clearance; proteoglycan.**

The small dermatan sulfate proteoglycans decorin and biglycan are ubiquitously distributed members of the family of small interstitial proteoglycans [see (1–3) for reviews]. Decorin, which has been studied more intensively, is synthesized as a prepro form, having 359 amino acids in the human. Its mature form consists of a core protein of 329 amino acids, a single glycosaminoglycan chain bound to serine residue 4, cystine loops near the N- and C-terminus, and either two or three asparagine-bound oligosaccharides (at asparagine residues 181, 232, and 273, respectively). The central part of the core protein consists of repeats of a leucine-rich sequence motif. Similar repeats in the ribonuclease inhibitor protein exhibit horseshoe-shaped structures with parallel β-sheets (4). Rotary shadowing–electron microscopy and molecular modelling studies suggested that decorin core protein is horseshoe shaped, too (5, 6). X-ray diffraction studies showed that in the case of ribonuclease inhibitor leucine-rich repeats on opposing sites of the horseshoe-like structure are involved in ligand binding (7), whereas the inner concave site of decorin was suggested to accomodate a single type I collagen triple helix (6).

Decorin is known to interact via its core protein with several components of the extracellular matrix. By binding to collagen types I, II, and VI it influences collagen fibrillogenesis and the final diameter of the fibrils (8–10). The biological importance of these interactions can be deduced from the phenotype of mice lacking the decorin gene (11). The animals are characterized by fragile skin, and their collagen fibrils have an uneven diameter, apparently due to uncontrolled lateral fusion. Interactions of decorin with fibronectin (12) and thrombospondin (13) lead to an inhibition of fibroblast adhesion to these substrata (13–15). Recently, a role of decorin in the control of cell proliferation has been proposed (16–18), and it has been hypothesized that decorin could serve as a reservoir of TGF-β[2] (19). From these observations it is evident that the regulation of the extracellular concentration of decorin by the rates

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[2] Abbreviations used: TGF-β, tumor growth factor beta; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LDL, low-density lipoprotein.

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of its biosynthesis and degradation is of great physiological importance.

Fibroblasts and other cells of mesenchymal origin are known to internalize decorin efficiently by receptor-mediated endocytosis (20–23). Two proteins of 51 and 26 kDa which are present in endosomes and at the plasma membrane (22, 24) have been considered as receptor proteins due to their high-affinity binding to the decorin core protein (22, 25). These proteins also interact with heparin and with highly sulfated domains in membrane-associated heparan sulfate (25, 26).

In this study we used recombinant decorin fragments and proteolytic degradation procedures to identify regions on the core protein which are involved in the interaction with the endocytosis receptor. Additionally, the influence of biglycan on decorin endocytosis was investigated.

MATERIALS AND METHODS

Cell culture and metabolic labeling. Human skin fibroblasts were grown in modified Eagle's minimum essential medium as described (22). For endocytosis experiments the $NaHCO_3$ concentration was reduced from 2.2 to 1.6 g/liter (endocytosis medium) to maintain a pH of 7.2 in an atmosphere of 95% air/5% CO_2. This pH of the culture medium is necessary for normal intralysosomal catabolism of glycosaminoglycans.

[^{35}S]Sulfate-labeled decorin was prepared from the media of human skin fibroblasts after incubation of the cells in the presence of 0.37 MBq/ml of [^{35}S]sulfate (Amersham-Buchler) for 72 h. Streptomycine sesquisulfate was omitted in these incubations, and fetal calf serum, which had been dialyzed against 0.15 M NaCl, was added to a final concentration of 4% (v/v). Purification of the proteoglycan was performed by anion-exchange chromatography on a Bio-Gel TSK DEAE-5 PW HPLC column (Bio-Rad) exactly as described (22). For reduction and alkylation decorin was incubated for 4 h at 37°C in 20 mM Tris/HCl, pH 8.6, containing 4 M guanidine hydrochloride and 20 mM mercaptoethanol. Iodoacetic acid (85 mM) was then added, and incubation continued for 30 min at 25°C in the dark. For mock treatment, mercaptoethanol and iodoacetic acid were omitted. Upon addition of fetal calf serum the samples were dialyzed against endocytosis medium.

Recombinant decorin core protein fragments expressed in prokaryotic cells. Recombinant fragments of human decorin were generated and purified as described recently (27). After renaturation, the fragments were dialyzed twice against 0.2× PBS and twice against 0.2× modified Eagle's minimum essential medium containing 1.6 g/liter $NaHCO_3$. Aliquots of 2 ml were lyophilized and stored under nitrogen. For use in endocytosis experiments, the fragments were reconstituted with 400 μl of H_2O, and any insoluble proteins were removed by centrifugation. Quantitation of the recombinant fragments was performed as described (27). All fragments used in this study could be reconstituted reproducibly as a 300 nM solution. At higher concentrations some of the fragments, among them Leu125–Val230, tended to become insoluble. The [^{35}S]cysteine/methionine-labeled peptide Asp15–Lys329 was prepared analogously after incubation of the induced bacterial cultures for 3 h in the presence of 0.37 MBq of [^{35}S]-sulfate (28).

Expression of recombinant decorin in human 293 kidney cells. Clone D6, which contains the complete coding region of human decorin core protein and had been described previously (29), served as the basis for the construction of plasmids allowing the expression of decorin constructs carrying glycosaminoglycan chains. An *Eco*RI/*Hpa*I fragment of clone D6 was ligated into the pUC18 vector (United States Biochemical) from which an *Eco*RI/*Xba*I fragment subsequently was cloned into pcDNA3 (Promega). This plasmid was used to allow the expression of wild-type decorin. Decorin with a deletion of the C-terminal amino acids Asp231–Lys329 was constructed as follows. Base pairs 457–898 of a *Hin*cII digest of the cDNA were cloned into the *Bam*HI site of pGem-4Z (Promega), thereby creating a leucine and a stop codon 3′ of the Val230 codon. Then, an *Eco*RI/*Alw*NI fragment of clone D6 was ligated with the *Alw*NI/*Xba*I fragment from pGem-4Z and cloned into pcDNA3 as above. The plasmid thus obtained was used for transfection. It served additionally as the template to create by PCR a decorin cDNA which exhibited, beside the C-terminal deletion, a deletion of the second to fourth leucine-rich repeats (Thr53–Thr121). In the first step 5′- and 3′-portions of the desired sequence were generated by using the primer pair 5′-CGCCAGTGTGCTGGAATTC-3′ (5′-sequence at the multicloning site) and 5′-GTCAGGGGGAAGATCCTTTG-3′ (reverse sequence of the 3′-end of the first leucine-rich repeat) and the primer pair 5′-CAAAGGATCTTCCCCCTGACCTTCAGGAGCTGCGTGCC-3′ (overlapping sequence of the 3′-end of the first and the 5′-end of the fifth leucine-rich repeat) and 5′-TATAGAATAGGGCCCTCT-AGA-3′ (reverse of the sequence at the 3′-end of the multicloning site), respectively. The amplified products were then subjected to a second PCR using the two primers designed from the multicloning site to yield a cDNA which could be ligated into the pcDNA3 vector after *Eco*RI/*Xba*I digestion. Sequencing of the constructs verified the presence of the desired deletions and the absence of point mutations in the remaining coding sequence.

Cultured 293 cells were transfected either by the calcium phosphate method or by Lipofectin (Gibco-BRL) and selected for neomycine resistance. Resistant cells were incubated in the presence of [^{35}S]-sulfate as above, and spent media were subjected to $(NH_4)_2SO_4$ and immune precipitation with a polyclonal, decorin-specific antiserum linked with protein A–Sepharose CL-4B (Sigma) as described (30). Bound proteoglycans were solubilized by adding 7 M urea in 20 mM Tris/HCl, pH 7.2, containing additionally 0.15 M NaCl and protease inhibitors (30). They were applied to a DEAE-Trisacryl M column (Serva) equilibrated with the same buffer and processed further, after application of urea-free buffer, as described for the preparation of [^{35}S]sulfate-labeled proteoglycans from the culture of fibroblasts.

Expression of recombinant biglycan in human 293 kidney cells. Biglycan cDNA was obtained from poly A$^+$ mRNA prepared from human osteosarcoma MG-63 cells. After reverse transcription a cDNA was obtained on the basis of the published human biglycan sequence (31). The forward primer contained additionally an *Eco*RI and the reverse primer a *Not*I restriction site. The forward primer had the sequence 5′-GTTGGAATTCGAGTAGCTGCTTTCGGTCC-3′ and the reverse-end complement primer the sequence 5′-ATAAGAATGCGG-CCGCCAAGGCTGGAAATGCATGAG-3′. After *Eco*RI/*Not*I digestion the cDNA was ligated into the pcDNA3.1(+) vector (Promega) and used for transfection of cultured 293 cells as described above. Neomycine-resistant cells were selected and shown to express biglycan carrying 2 and 1 glycosaminoglycan chains, respectively. The proteoglycan was purified to homogeneity, as judged by SDS–PAGE, by successive chromatographies on DEAE-Trisacryl M and phenyl-Sepharose (Sigma). Details of the procedure will be given elsewhere (G. Narayek, M. Müller, and H. Kresse, manuscript in preparation).

Immunization and preparation of antibody Fab fragments. The recombinant Leu125–Val230 fragment was purified from transformed bacteria as described and renatured in the absence of bovine serum albumin. The fragment (75 μg) was dissolved in phosphate-buffered saline, pH 7.4, and the solution was mixed with an equal volume of Freund's adjuvant, either complete (for the first injection) or incomplete (for booster injections). The mixtures were injected into a rabbit intracutaneously on days 0, 18, and 30. Sera were collected 7 and 10 days after the booster injections.

Antibodies specific for decorin were isolated by two successive affinity-purification steps. In a first step, antiserum was applied to an affinity column with recombinant biglycan core protein (28) immobi-

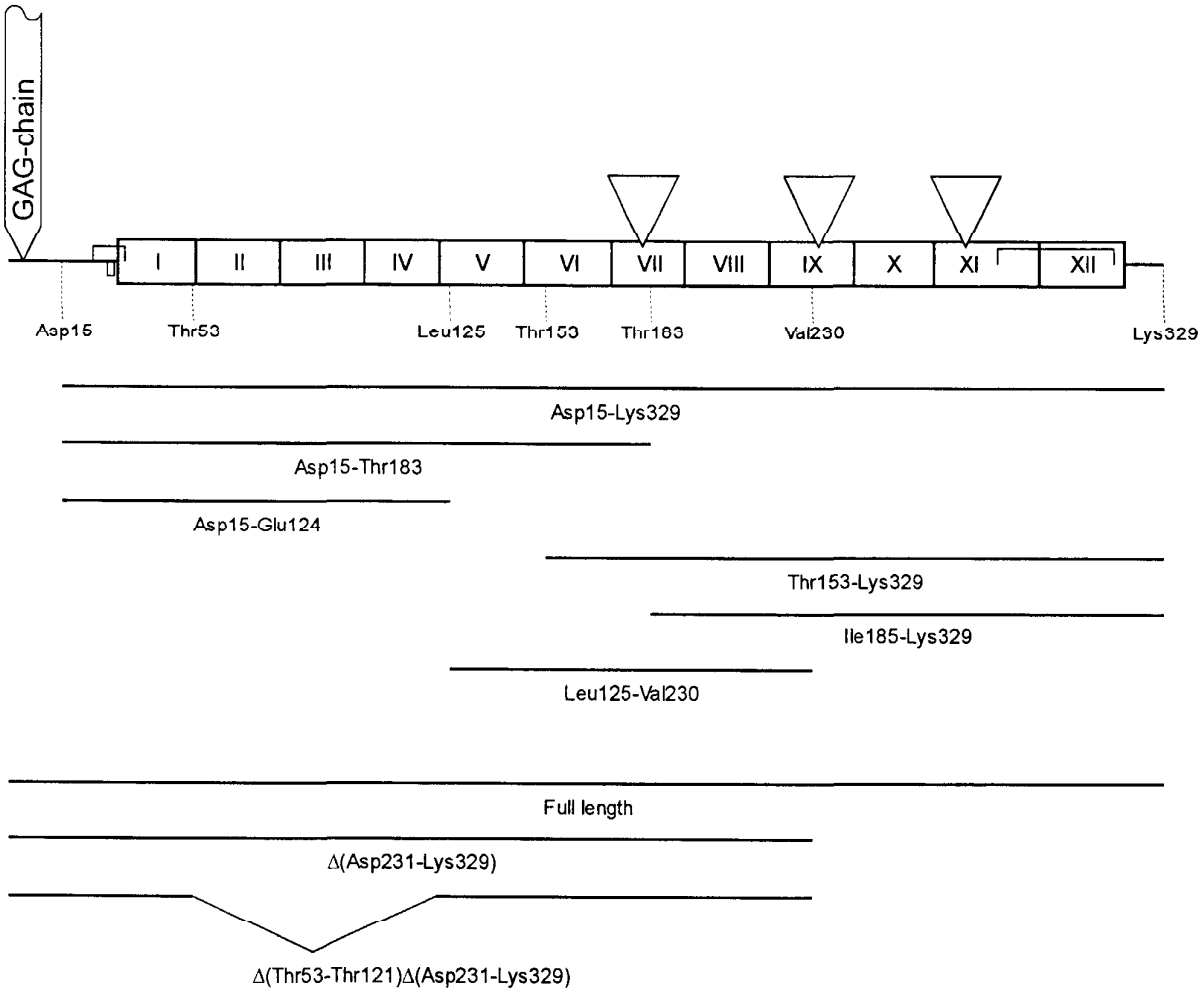

FIG. 1. Schematic representation of mature decorin core protein. The leucine-rich repeats are indicated by roman numerals, N-glycosylation sites by triangles, and disulfide loops by brackets.

lized to CNBr-activated Sepharose to remove antibodies recognizing the fusion protein part of the recombinant molecules. In a second step, the breakthrough of the first affinity column was applied to a column with immobilized recombinant decorin core protein. After washing the column with 20 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 0.01% Tween 80, bound antibodies were eluted with 0.1 M glycine, pH 3.0.

For digestion with papain, the eluate was dialyzed against 0.1 M sodium acetate buffer, pH 5.5. The protein concentration was adjusted to 2 mg/ml, and the solution was made 50 mM with cysteine and 1 mM with EDTA and incubated in the presence of 10 μg papain/mg antibody at 37°C for 9 h. Papain was then inactivated by adding iodoacetamide to a final concentration of 75 mM and incubation at ambient temperature for 30 min. Completeness of the digestion was confirmed by SDS–PAGE. Fc fragments were removed by passing the solution, after dialysis against phosphate-buffered saline, over a column of protein A–Sepharose.

Endocytosis. Endocytosis of [^{35}S]sulfate-labeled decorin was measured as described (22). Since endocytosis of decorin is followed by intralysosomal degradation, and the resulting products are in part released into the culture medium, endocytosis is represented by the sum of the intracellular radioactivity and the ethanol-soluble radioactivity in the culture medium. Degradation is defined as the sum of intra- and extracellular ethanol-soluble radioactivity over the total amount of endocytosed material. For comparison, endocytosis is usually expressed as clearance rate, giving the volume of incubation medium (in microliters) cleared from the labeled material per hour and per milligram cell protein.

Other methods. Biotinylation of native decorin and of the recombinant Leu125–Val230 fragment with diazobiotin (Boehringer Mannheim), which introduces biotin residues at histidine and tyrosine residues of the protein molecule, was performed according to the instructions of the manufacturer. Enzymatic digestion of decorin with chondroitin ABC lyase (31), SDS–PAGE (32), and Western blotting (34) and the purification of the endocytosis receptor from rat brain (24) were performed as described. Bound biotinylated ligands were visualized on Western blots by an enhanced chemiluminescence reaction (Amersham-Buchler) as described in the manufacturer's protocol after incubating the blots with peroxidase-conjugated Extr-Avidin (Sigma). Endoproteinase Lys-C-fragments of decorin were obtained by incubating 200 μg of decorin with 2 μg of the protease (Sigma) in 25 mM Tris/HCl, pH 8.5,

TABLE I

Inhibition of Decorin Endocytosis by Recombinant Decorin Core Protein Fragments

Competitor (100 nM)	Inhibition (%)
Asp15–Lys329	43.5 ± 4.9 (n = 4)
Asp15–Thr183	40.0 ± 3.6 (n = 3)
Asp15–Glu124	22.7 ± 2.6 (n = 4)
Thr 153–Lys329	35.8 ± 2.8 (n = 4)
Ile185–Lys329	29.1 ± 2.6 (n = 4)
Leu125–Val230	35.5 ± 5.4 (n = 5)

Note. Human skin fibroblasts were incubated for 3 h with [^{35}S]-sulfate-labeled decorin (1.5–2.7 × 10^5 cpm/dish in the different experiments). The clearance rates in the absence of competitor were 30.0 ± 4.8 μl × h^{-1} × mg^{-1} (n = 5), and 100 nM wild-type decorin from fibroblast secretions caused an inhibition by 56 ± 12% (n = 5). The data were collected from 3–5 independent experiments using the same peptide preparations.

containing 1 mM EDTA, for 18 h at 37°C. After inactivation of the enzyme by 200 μg of leupeptin, the glycosaminoglycan chain-bearing N-terminal fragment was removed by adsorption to DEAE-Trisacryl M. LDL uptake was followed by measuring the intracellular cholesterol concentration (cholesterol kit, Boehringer Mannheim, Germany). LDL was kindly provided by Dr. A. von Eckardstein, University of Münster.

RESULTS

Influence of Reduction and Alkylation

Human skin fibroblasts were incubated for 6 h in the presence of 3 × 10^4 cpm of [^{35}S]sulfate-labeled decorin which had been reduced and alkylated or subjected to mock treatment. The clearance rates were 77 μl/h/mg cell protein for the control and 105 μl/h/mg protein for the reduced and alkylated sample. This indicates that the disulfide bonds between the cysteins 24 and 30, 28 and 36, and 258 and 291 are not involved in establishing the tertiary structure required for interaction with the endocytosis receptor.

Inhibition of Decorin Endocytosis by Recombinant Decorin Fragments

The N-terminal portion of decorin shows the greatest variability among different species (35). However, human decorin was endocytosed efficiently by cultured human, bovine, and rat mesenchymal cells (bovine smooth muscle cells, rat mesangial cells, human and rat skin fibroblasts). Three comparative experiments yielded clearance rates of at least 35 μl/h/mg cell protein. Such clearance rates are indicative of receptor-mediated uptake and suggest that the structure recognized by the receptor resides in a more conserved part of the ligand.

In the search for receptor binding sites recombinant decorin peptides were therefore generated which in all cases lacked the N-terminal 14 amino acids of the mature core protein and were truncated in other N- and/or C-terminal regions. Their structure can be deduced from Fig. 1. The peptides were employed as potential inhibitors of uptake of [^{35}S]-sulfate-labeled decorin which was obtained from human skin fibroblasts. Doses of up to 3 × 10^5 cpm/dish of the radioactive proteoglycan were applied. It had been shown before that within this range of doses uptake is related linearly to the quantity of applied decorin (22, 23). Table I shows the results of several independent experiments using the same peptide preparations. Some variations were observed with different preparations of recombinant peptides. However, the relationship between the different fragments was similar in all experiments performed. The most potent inhibitor was the Asp15–Lys329 fragment, being almost as efficient as wild-type decorin. Interestingly, all other recombinant peptides containing only some leucine-rich repeat structures, were also inhibitory. However, the three fragments having in common the sequence Thr153–Thr183 (Asp15–Thr183, Thr153–Lys329, Leu125–Val230) were most effective, and the weakest inhibitory potency was found for fragment Asp15–Glu124. The central Leu125–Val230 fragment was most often almost as effective as inhibitor as the Asp15–Lys329 fragment, but was occasionally ineffective. This variability is probably due to an occasionally ineffective renaturation of this particular fragment.

To study directly the uptake of recombinant decorin, expressed in prokaryotic cells, a [^{35}S]cysteine/methionine-labeled recombinant Asp15–Lys329 fragment was prepared. It was cleared efficiently from the medium (Table II). The high clearance rate obtained should be judged in light of the observation that the substitution of the core protein with a dermatan sulfate chain and with N-linked glycans negatively influences the uptake properties (21). In support of the conclusions drawn from the data of Table I, native decorin from the culture media of fibroblasts, the unlabeled recombinant Asp15–Lys329 fragment as well as Leu125–Val230 exhibited a similar inhibitory effect

TABLE II

Endocytosis of the [^{35}S]Cys-, Met-Labeled Recombinant Decorin Fragment Asp15–Lys329

Competitor (100 nM)	Inhibition (%)
Wild-type decorin	30.1
Asp15–Lys329	26.5
Leu125–Val230	25.0

Note. The clearance rate in the absence of competitor was 136 μl × h^{-1} × mg^{-1}.

TABLE III

Endocytosis of [^{35}S]Sulfate-Labeled Recombinant Decorin Species Expressed in 293 Cells

Recombinant decorin	Clearance rates ($\mu l \times h^{-1} \times mg^{-1}$)	Degradation (% of endocytosed amount)
Full length	8.2	72
Deletion Asp231–Lys329	6.8	82
Deletions Thr53–Thr121 and Asp231–Lys329	7.1	75

Note. Values for endocytosis are the mean of two parallel experiments. The clearance rate for wild-type decorin from fibroblast secretions was 16.9 $\mu l \times h^{-1} \times mg^{-1}$.

on the uptake of the labeled Asp15–Lys329 decorin fragment.

Endocytosis of Recombinant Decorin Expressed in Eukaryotic Cells

The data presented so far did not allow the assignment of a short stretch of amino acids as binding site for the decorin endocytosis receptor, although they suggest the importance of leucine-rich repeat structures in the central part of the molecule. However, all recombinant decorin constructs expressed in prokaryotic cells were devoid of asparagine-bound oligosaccharides and of the glycosaminoglycan chain. For studying decorin constructs expressed in eukaryotic cells, 293 cells were transfected with decorin cDNAs encoding the full-length core protein and truncated fragments either lacking the C-terminus with its three leucine-rich repeates alone (deletion of Asp231–Lys329) or lacking additionally three N terminally located leucine-rich repeats (Thr53–Thr121). The latter construct most closely resembles the prokaryotic fragment Leu125–Val230. The recombinant proteoglycans were secreted into the culture medium which implies that the constructs were folded in a manner suited for intracellular transport and glycosaminoglycan attachment. Human 293 cells do not express decorin themselves (36). The recombinant decorin species could, therefore, be purified by immunoprecipitation which yielded pure preparations (as judged by SDS–PAGE, data not shown) but required an intermittent denaturing step to dissociate the immune complex.

When these constructs were used for endocytosis they were cleared with similar efficiency by cultured fibroblasts and were degraded intralysosomally to a similar extent (Table III). Furthermore, within the dose range employed (up to 25,000 cpm/dish), there was a linear relation between applied dose and uptake. However, all the recombinant decorin species were endocytosed less efficiently than decorin from skin fibroblasts subjected to the same purification protocol. Since the composition of the N-linked oligosaccharides is known to influence the uptake properties (21) it is tempting to postulate a different oligosaccharide structure of decorin from fibroblasts and 293 cells, respectively.

Inhibition of Decorin Endocytosis by Fab Fragments of Anti-Leu125–Val230 Antibodies

To obtain independent proof of the direct involvement of central structures of the decorin core protein for receptor-mediated endocytosis, antibodies against the recombinant Leu125–Val230 fragment were raised, and Fab fragments prepared from these antibodies were used to study their potential inhibitory effect on decorin endocytosis. It is shown in Fig. 2 that these Fab fragments were indeed able to inhibit the endocytosis of [^{35}S]sulfate-labeled decorin by human skin fibroblasts in a dose-dependent manner. Complete inhibition of endocytosis, however, could not be achieved at the highest dose used.

Binding of the Leu125–Val230 Fragment to the Endocytosis Receptor

Provided that the central part of decorin core protein encompasses structures suited for receptor-mediated endocytosis, fragment Leu125–Val230 should be able to bind to the endocytosis receptor, a 51-kDa protein. For a direct demonstration of such a binding, fractions from a heparin-affinity chromatography of a rat brain extract containing the decorin endocytosis receptor protein were separated by SDS–PAGE and blotted onto nitrocellulose. The blot was incubated with biotinylated probes of native decorin, decorin core protein obtained by digestion of native decorin with chondroitin

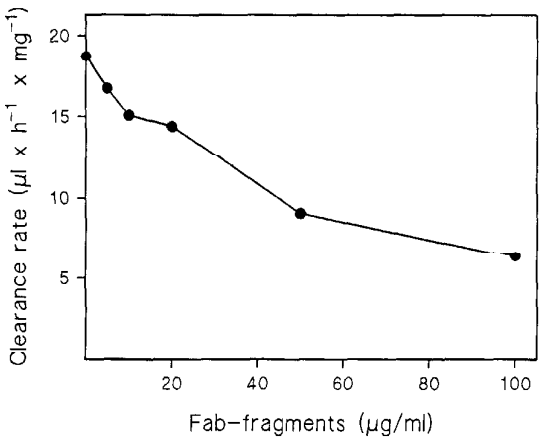

FIG. 2. Inhibition of decorin endocytosis by Fab fragments of antibodies against the recombinant decorin fragment Leu125–Val230. Human skin fibroblasts were incubated for 4 h with native [^{35}S]-sulfate-labeled decorin (150,000 cpm/dish) in the presence of the indicated amounts of Fab fragments of antibodies.

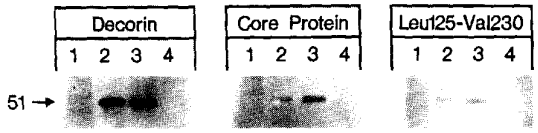

FIG. 3. Binding of decorin, decorin core protein, and recombinant Leu125–Val230 fragment to the 51-kDa decorin endocytosis receptor. A rat brain extract was partially purified and subjected to heparin–Sepharose affinity chromatography. Fractions eluting at 350 mM NaCl (1), 400 mM NaCl (2), 450 mM NaCl (3), and 1.5 M NaCl (4) were separated by SDS–PAGE, blotted onto a nitrocellulose membrane, and incubated with the biotinylated ligands as indicated. Bound ligands were visualized by chemiluminescence after incubation with a peroxidase-conjugated avidin derivative.

ABC lyase, or the recombinant Leu125–Val230 fragment. As expected, binding of the recombinant Leu125–Val230 fragment to the 51-kDa endocytosis receptor could be demonstrated (Fig. 3).

Influence of Endoproteinase Lys-C-Generated Peptides

The capability of peptide Leu125–Val230 to interfere with decorin endocytosis by direct interaction with the receptor could be established clearly. Smaller peptides were generated by endoproteinase Lys-C treatment of decorin from fibroblasts and tested for their interference with decorin endocytosis. The protease leaves the 7th leucine-rich repeat (Leu172–Ser192) intact and yields as larger, N-glycosylated fragment Leu172–Lys202. These peptides did not serve as inhibitors of decorin endocytosis at concentrations between 60 and 900 nM (result not shown) whereas the recombinant peptides were inhibitory at a concentration of 100 nM (Table I).

Competition of Decorin Endocytosis by Biglycan

For further studies on the structural requirements for receptor-mediated endocytosis we tested the influence of recombinant biglycan expressed in human 293 cells on decorin endocytosis. In its central structure, Val106–Ile213 (31), which is the sequence corresponding to Leu125–Val230 in decorin (2), biglycan is 47% identical with decorin. For control purposes recombinant full-length decorin from 293 cells was also tested. At a dose of 135 nM biglycan the uptake of [^{35}S]sulfate-labeled decorin from fibroblasts was inhibited by 48%, whereas the same dose of decorin caused an inhibition by 57%. At doses of 1.35 μM the inhibition was 87% (biglycan) and 79% (decorin), respectively. Thus, biglycan exhibited the same inhibitory potency on decorin uptake as decorin itself.

A Dixon plot of the dose-dependent inhibition of decorin uptake by biglycan suggested a partially competitive inhibition mechanism (Fig. 4). This finding should be judged in the context of a weak interaction of protein-free dermatan sulfate with the endocytosis receptor.

Leucine-rich repeat structures themselves do not inhibit the endocytosis of unrelated molecules. A fourfold molar excess of decorin had no inhibitory effect on LDL uptake (concentration 100 μg/ml) by fibroblasts (data not shown).

DISCUSSION

It had been shown previously that decorin core protein is required for efficient internalization and must, therefore, carry one or several recognition sites for the interaction with its endocytosis receptor (21). The data

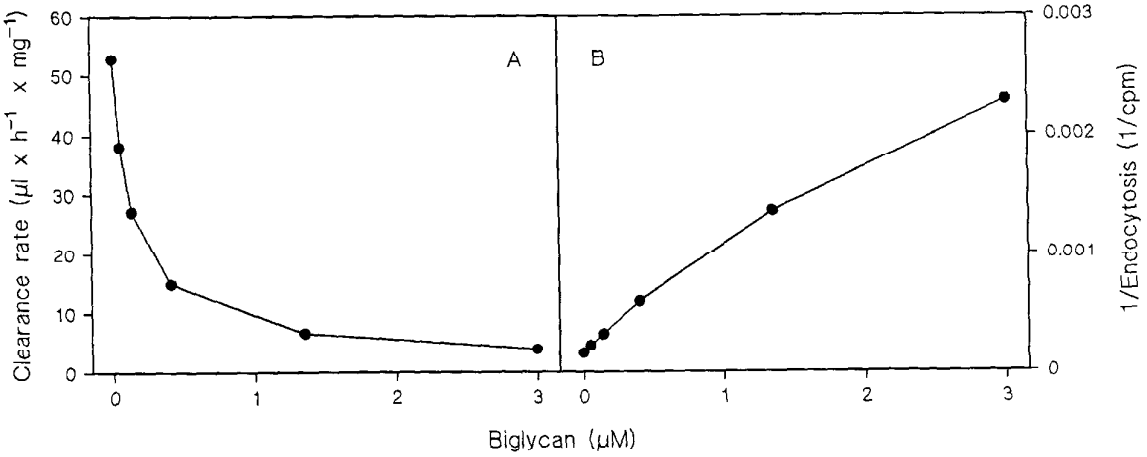

FIG. 4. Inhibition of decorin endocytosis by recombinant biglycan from 293 cells. Human skin fibroblasts were incubated for 3 h with [^{35}S]sulfate-labeled decorin from fibroblast secretions (250,000 cpm/dish) in the presence of the indicated concentrations of biglycan which were calculated on the basis of core protein quantification. Decorin clearance rates are shown in (A), and a Dixon plot of the data from the same experiment is given in (B).

presented in this paper strongly suggest that structures within the central part of the core protein contain sufficient information for a biologically effective interaction with the endocytosis receptor. On the contrary, Asp1–Arg14 and Asp231–Lys329 are not required for receptor-mediated uptake of decorin. A single specific sequence motif, however, could not be assigned as a unique binding site. Various recombinant core protein fragments comprising most of the leucine-rich repeat structures, which are known to be responsible for protein–protein interactions, exhibited inhibitory activity for the endocytosis of wild-type decorin from skin fibroblasts. Similarly, full-length recombinant biglycan appeared to interact as efficiently with the receptor as recombinant decorin. It is conceivable that the binding properties of the endocytosis receptor for decorin (and biglycan) depend to a great extent on the regular spacing of conserved amino acid residues which are present in most or all leucine-rich repeat structures. This may be in contrast to the interactions between decorin and type I collagen where the sixth leucine-rich repeat with a conserved glutamate residue seems to be of special importance (27, 37). A single binding site probably does not exist for the decorin endocytosis receptor. In light of the results presented in this study one may assume that more than one structure within the leucine-rich repeats exhibits sufficient affinity for the endocytosis receptor. A preferential, albeit not exclusive, binding site appears to be located within the Leu125–Val230 region. The importance of this region is supported by several independent investigations: (i) by the results of competition experiments, (ii) by the direct demonstration of uptake and degradation of glycosylated decorin constructs truncated in regions at the C-terminus and near the N-terminus, (iii) by the direct demonstration of binding of Leu125–Val230 to the receptor, and (iv) by the effective inhibition of decorin endocytosis in the presence of Fab fragments directed against this region. Biglycan, which is only 47% identical in the corresponding core protein region, nevertheless exhibited an inhibitory activity similar to that of decorin.

The preparation of truncated decorin molecules—either from prokaryotic or eukaryotic cells—involved denaturation and renaturation steps which may have an unknown influence on the tertiary structure of the molecules (38). Unfortunately, the presence of albumin required for the renaturation of the peptides from prokaryotic cells and the small quantity of proteoglycans derived from 293 cells precluded investigations by CD spectroscopy. However, it has been shown previously that wild-type decorin and biglycan can be taken up by receptor-mediated endocytosis after denaturation and renaturation (23). Furthermore, the inhibitory effect of the antibodies against Leu125–Val230 on the uptake of wild-type decorin which had never been exposed to denaturing conditions represents an independent argument for the importance of the central leucine-rich repeat structures.

In summary, the data presented in this paper suggest that a preferential decorin binding site for its endocytosis receptor is located within the central leucine-rich repeat structure of the core protein. It appears that there may be several appropriate binding structures along this sequence, but a major binding site is proposed to be located in the Leu125–Val230 region.

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